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A Developmental Perspective: Changes in the Position of the Blastopore during Bilaterian Evolution

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Progress in resolving the phylogenetic relationships among animals and the expansion of molecular developmental studies to a broader variety of organisms has provided important insights into the evolution of developmental programs. These new studies make it possible to reevaluate old hypotheses about the evolution of animal body plans and to elaborate new ones. Here, we review recent studies that shed light on the transition from a radially organized ancestor to the last common ancestor of the Bilateria ("Urbilaterian") and present an integrative hypothesis about plausible developmental scenarios for the evolution of complex multicellular animals.

The Bilaterian Ancestor

Evolutionary developmental biologists have attempted to understand the molecular basis for differences in the organization of animal body plans and to generate plausible, testable scenarios for how these molecular programs could be modified to give rise to novel forms. Most of this work has focused on a monophyletic group of triploblastic animals, the Bilateria: animals that possess an anterior-posterior axis and a dorsoventral axis that define a plane of bilateral symmetry. In addition to derivatives of ectoderm (skin and nervous system) and endoderm (gut and its derivatives), triploblastic animals have derivatives of the third "middle" germ layer called mesoderm, which includes musculature, the circulatory system, excretory system, and the somatic portions of the gonad. Bilaterians have historically been divided into two major evolutionary groups (Figure 1): the deuterostomes (which includes vertebrates like human beings) and the protostomes (which includes the majority of other invertebrate animals, including the developmental model systems *C. elegans* and *Drosophila*). These groups were named over 100 years ago and were defined on the basis of embryological principles. Typically in deuterostomes, the position in the embryo that gives rise to endodermal tissues (called the blastopore) at the onset of gastrulation gives rise to the anus of the adult animal. The mouth of deuterostomes ("secondary mouth") forms at a different location. In the last common ancestor of all protostomes ("mouth first"), the site of gastrulation was said to give rise, not to the anus, but to the adult mouth. These terms are a testament to our recognition of the importance of changing patterns of developmental patterns in the generation of body plan diversity during organismal evolution.

Reconstructing molecular and morphological characteristics of the "Urbilaterian" (the last common ancestor of all bilaterians) has been a central goal in evolutionary developmental biology. Fueling this effort is the fact that most of the available information about the cellular and molecular details of development is gleaned from a handful of genetic models systems (Figure 1) such as mice (deuterostomes) and flies and nematodes (protostomes). These systems revealed some shared developmental molecular mechanisms between protostome and deuterostomes, leading to the idea that their last common ancestor was a complex organism with a reiterated, segmented body plan, a central nervous system with an anterior brain, a through gut with a ventral mouth, and a mesodermally derived circulatory system and coelom (body cavity) (see e.g., De Robertis and Sasai, 1996). However, the most recent phylogenetic arguments that incorporate a much greater spectrum of the existing biological diversity (Figure 1) demonstrate that these morphological features are not likely characteristics of the Urbilaterian (Baguñá and Riutort, 2004; Hejnol and Martindale, 2008b) and may not even represent the protostome-deuterostome ancestor (Lowe et al., 2003, 2006). Our deeper and more accurate understanding of the evolutionary relationships among animals no longer justifies the assumption that the deuterostome ancestor resembled a vertebrate chordate or that the protostome ancestor resembled a dipteran arthropod (Arendt and Nübler-Jung, 1997; Carroll et al., 2001; De Robertis, 2008; De Robertis and Sasai, 1996). Indeed, while many scientists assume that the relationships among living animals, the order, time, and position in which they arose, and hence the origin of distinct morphological features during evolution, have already been solved, this is not the case. Molecular approaches have, and continue to, radically change our understanding of animal evolution with profound implications regarding the direction of evolutionary change (see, e.g., Arendt and Nübler-Jung, 1994; Arendt et al., 2001; Denes et al., 2007; Finnerty et al., 2004; Hejnol and Martindale, 2008a; Lowe et al., 2006).

It has only been a decade (Aguinaldo et al., 1997) since we realized that flies (arthropods) and nematodes are related to one another in a group called the Ecdysozoa (Figure 1), but these animals do not look similar. Did the common ancestor of these two groups have reiterated body segments, a mesodermally lined body cavity (called a coelom), and lateral appendages that were lost in the lineage that gave rise to the nematodes? Or, did these morphological features evolve independently in

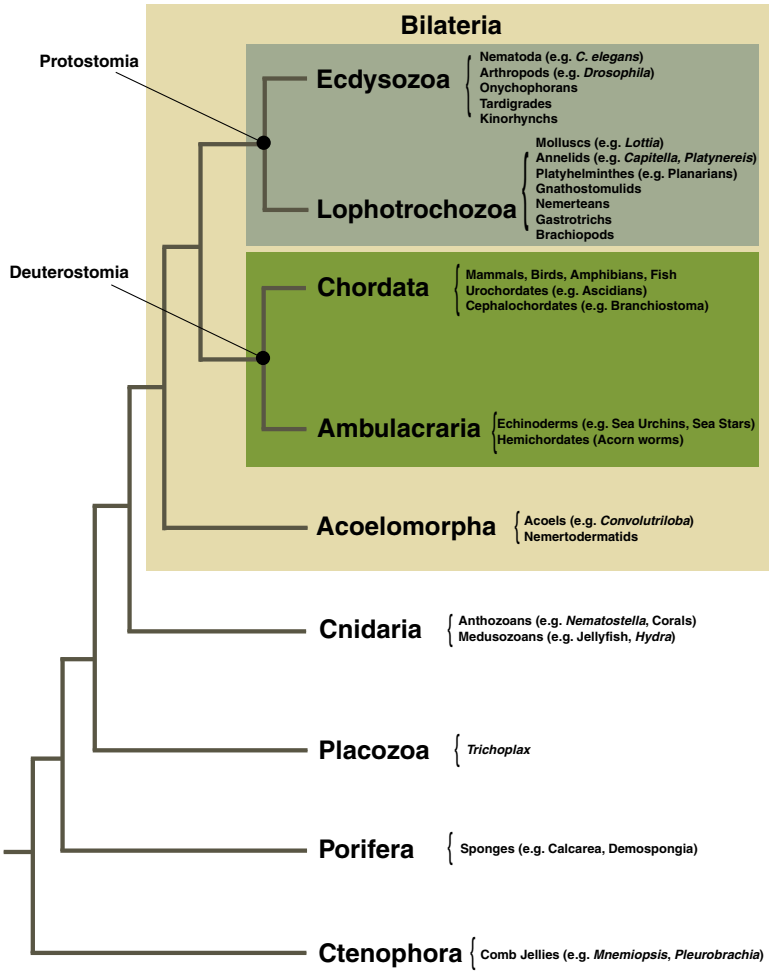

Figure 1. Phylogenetic Relationships of the Metazoa with Representative Taxa and Species Listed

The animal phylogeny is based on Dunn et al., (2008) and Paps et al. (2009). The position of ctenophores is still controversial (see Philippe et al., 2009).

the arthropod line? In order to determine the features of the ecdysozoan ancestor, one also needs to know what the common ancestor of the lophotrochozoan group (Figure 1), which gave rise to animals as diverse as planarians, snails, and squids, looked like. And to understand the protostome ancestor, we need to know what the deuterostome ancestor that gave rise to sea urchins, sea squirts, and vertebrates was like.

All these questions need to be answered first, before one can reconstruct the last common ancestor of the Bilateria, the iconic Urbilaterian. Recent phylogenetic studies incorporating a larger diversity of animal groups suggest that acoelomorph flatworms (Figure 1) are likely to share characteristics in common with the Urbilaterian (Ruiz-Trillo et al., 2004; Ruiz-Trillo et al., 1999; Telford et al., 2003; Wallberg et al., 2007). Acoelomorphs are small, direct developing (no larval form), unsegmented, ciliated, appendage-less worms. They have definitive mesoderm that forms muscle (but no coelom, circulatory, or excretory system), multiple parallel longitudinal nerve cords (i.e., no dorsally or ventrally "centralized" nervous system), and a single opening to the gut cavity (Bourlat and Hejnol, 2009; Haszprunar, 1996; Rieger et al., 1991). Animal groups that arose even earlier in metazoan evolution than bilaterians (Figure 1), such as the cnidarians (sea anemones, corals, and jellyfish), ctenophores (comb jellies), and sponges, also lack a through gut (with separate mouth and anus), a coelom, appendages, excretory system, reiterated body segments, and a (dorsally or ventrally) centralized nervous system, suggesting that the common ancestor of all bilaterian groups may have also lacked these features and raising the possibility that these morphological characteristics arose at least once, or perhaps multiple times, during metazoan diversification.

These results illustrate a renewed importance of mapping morphological characters on to robust phylogenetic trees to determine the direction of evolutionary change. Total genome sequencing from a growing list of metazoans (e.g., Putnam et al., 2007, 2008; Srivastava et al., 2008) has shown that there is no simple relationship between genomic/molecular complexity and organismal/developmental complexity, so the mere presence of members of conserved gene families (e.g., "segmentation genes") reveals little about how they were deployed at different nodes of animal evolution. Understanding the true history of phylogenetic relationships of metazoan animals is thus of utmost importance for understanding the history of life on planet Earth because it could reveal whether certain features (e.g., coeloms, body segments, nerve cords, digestive system, etc.) previously thought to have characterized the Urbilaterian evolved independently in different animal lineages. Thus, a detailed understanding of the developmental basis for the formation of these structures in all different metazoan lineages is essential for understanding how molecular pathways were modified to generate the vast array of biological diversity in existence.

The Radial to Bilateral Transition: Major Hypotheses

While the complexity and modifications of organs and organ systems dominate discussion of bilaterian evolution, less attention has been focused on the initial evolutionary origin of these traits. Arguably the most profound change in body plan organization in the Metazoa occurred in the early animal lineages that gave rise to the Bilateria, where important traits like mesoderm, a condensed nervous system, and a clear bilateral body axis appeared from a morphologically much simpler animal (Schmidt-Rhaesa, 2007). Several scenarios formulated by different authors of the last century attempt to explain such a transition and try to combine the evolution of the body axes with the evolution of complex organ systems in the context of life history evolution. Virtually all scenarios that explain the evolution of the bilaterian body plan are versions of Haeckel's "Gastraea" theory (Haeckel, 1874), which posits a simple diploblastic organism composed of an ectodermally derived epidermis surrounding an endodermally derived blind gut with a single posterior opening to the outside

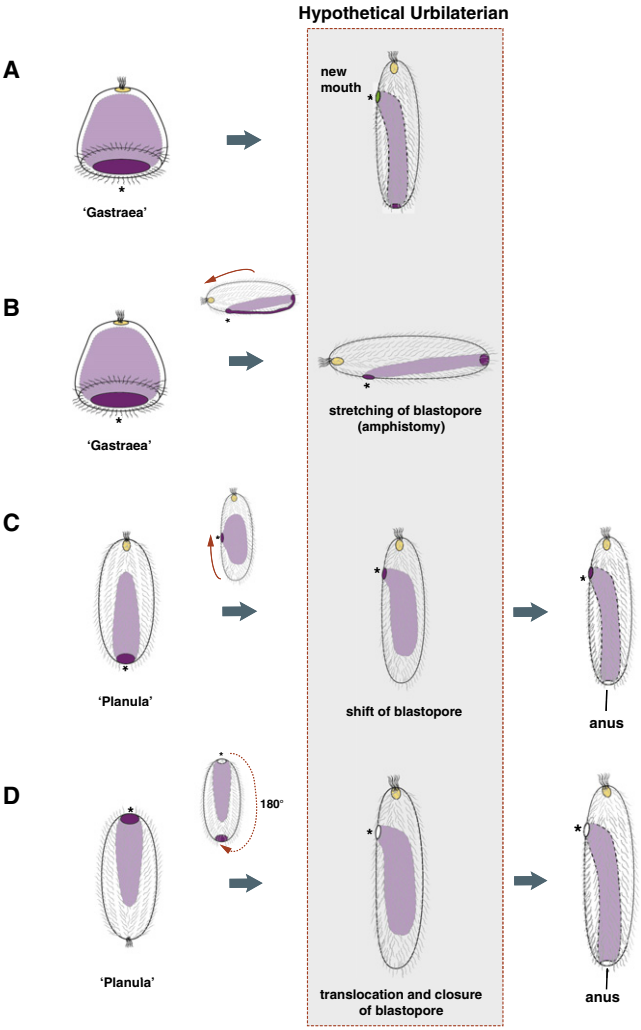

Figure 2. Hypothesis about Bilaterian Body Plan Evolution from a Radially Symmetrical Ancestor

Various scenarios explaining the transition to bilaterality during animal evolution emphasizing the relationship between the position of the mouth (marked with an asterisk), anus, and the site of gastrulation (purple shading), with respect to the primary egg axis (derivatives of the animal pole up). Note that the anterior sensory apparatus (yellow shading) is assumed to form in the same position (anterior) in all metazoans. The gut is shaded light purple.

(A) The posterior site of gastrulation and original mouth of the ancestral Gastraea forms the anus of modern animals, with a new mouth (green) forming anteriorly. This scenario closely resembles the modern day embryonic process of deuterostomy.

(B) The posterior site of gastrulation and original mouth represent the ventral surface of modern animals. The mouth and anus of the modern day through gut form simultaneously by a process called amphistomy. The apical sensory organ becomes the brain and migrates to new anterior pole (red arrow).

(C) The acoel-planuloid hypothesis predicts that the posterior mouth migrates anteriorly along the ventral surface over evolutionary time (red arrow). The anus forms secondarily with no formal relationship to the site of gastrulation.

(D) An alternative hypothesis based on embryological and molecular evidence in which the mouth forms from oral ectoderm derived from the animal hemisphere and the site of gastrulation (red) moves from the ancestral animal (ctenophores and cnidarians) to vegetal pole (Bilateria). Note that the sensory organs of ctenophores and cnidarians are convergent condensations of nervous elements and not homologous to metazoan anterior neural structures. According to this hypothesis, all oral openings are homologous across the Metazoa (except chordates) and became dissociated from the position of the blastopore in bilaterians. The anus evolved after the mouth, possibly independently in different lineages, and bears a strict relationship to the blastopore only in deuterostomes.

world (e.g., mouth/anus) as the hypothetical precursor to all bilaterian forms. The anterior-posterior axis of this ancestral animal is defined by the direction of locomotion (e.g., the major swimming or crawling axis), with the differentiated neural/sensory structures at the leading pole being homologous with the anterior brain of extant bilaterians (Figure 2).

A critical issue related to the developmental explanation for how more complex bilaterians arose from a bilayered organism is the site of gastrulation (the spatial position of presumptive endodermal gut tissue) and its relationship to the original opening to the gastric cavity of an ancestral metazoan relative to the direction of locomotion (Figure 2). In one scenario, the site of gastrulation (blastopore) of the ancestor remains as the posterior opening to the digestive tract (anus), with a new mouth evolving independently from an opening anteriorly (Figure 2A; see Lankester, 1877). This developmental pattern is called deuterostomy and is seen in extant members of a large clade of animals including echinoderms, hemichordates, cephalochordates, and vertebrates (Deuterostomia) (Figure 1). Another idea, the Acoeloid-Planuloid hypothesis (Von Graff, 1891), suggests that the blastoporal opening to blind gut originally occurred in the posterior region but then moved anteriorly along the ventral surface over evolutionary time (Figure 2C). This idea argues that the mouth is homologous in all animals and that the formation of a second opening to the gut, the anus, occurred secondarily (Figure 2) (Beklemishev, 1969; Hyman, 1951; Salvini-Plawen, 1978). A third hypothesis argues that a posterior opening to the gut of a "Gastraea"-like ancestral creature gives rise to both mouth and anus simultaneously by a process called amphistomy, in which a slit-like elongation of the blastopore followed by a lateral closure gives rise to openings at both ends of the through gut (Figure 2B) (Arendt and Nübler-Jung, 1997; Malakhov, 2004; Remane, 1950; Sedgwick, 1884). Clearly, these theories cannot all be correct and each has a distinct set of predictions relative to the developmental basis for bilaterian body plan evolution.

All these theories rely heavily on observations of cnidarian development, the well-accepted sister group to the Bilateria (Figure 1). Cnidarians possess a swimming ciliated planula stage (Figure 3) in which the site of gastrulation (and the future mouth opening) is located at the posterior (trailing) pole of the swimming direction and a neural structure (called the apical tuft), tacitly assumed to be homologous with the bilaterian brain (Nielsen, 1999, 2005a), is located at the leading end. We will demonstrate in this review that most of these concepts of metazoan evolution are not consistent with the developmental data recently obtained from diverse animals. While the previous hypotheses were based on larval morphology and the swimming/crawling direction as the major axial organizing system, we here argue that developmental phenomena organized around the animal-vegetal axis delivers more insights into the developmental modifications that lead to evolutionary transitions of body plan organization in the stem lineage of the Bilateria.

A Developmental Perspective: The Importance of the Primary Egg Axis

All metazoan embryos arise from products of meiosis. Oogenesis and spermatogenesis are among the unifying apomorphies for the Metazoa (Ax, 1996). In the oocyte, the position where the

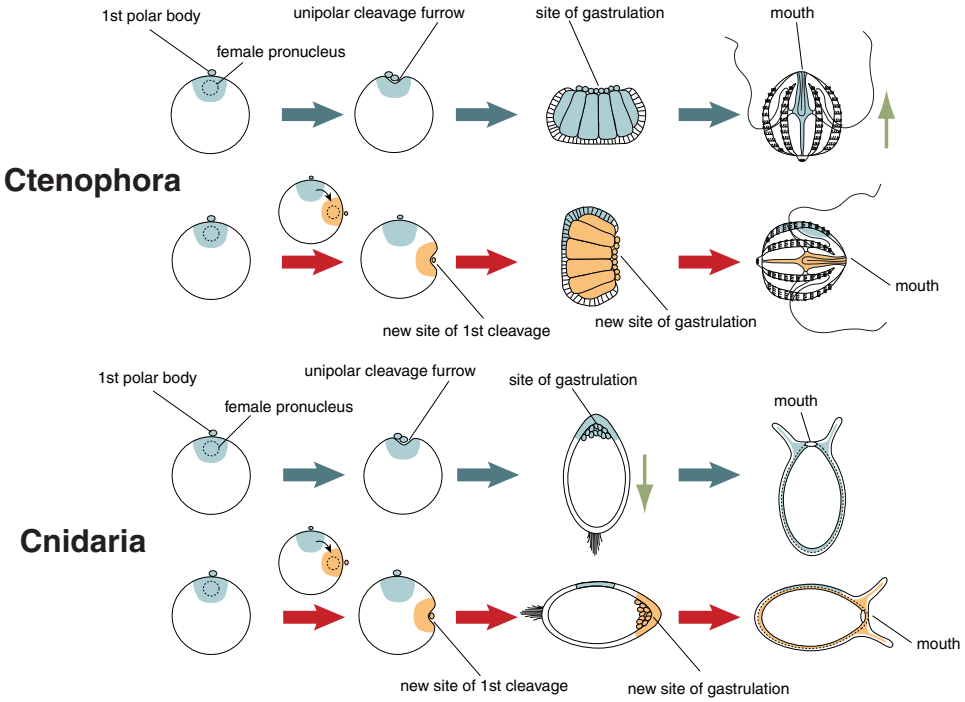

Figure 3. Fate Mapping Experiments Indicating the Origin of the Oral Pole in Ctenophores and Cnidarians
Vital dye labeling of the animal pole (indicated by the position of the polar bodies) in ctenophore and cnidarian oocytes shows that the site of the unipolar first cleavage furrow becomes the site of gastrulation (endomesoderm formation). In both ctenophores and cnidarians, when the meiotic nucleus and its surrounding cytoplasm is translocated to an ectopic site by centrifugation (experiment with red arrows), it induces a new site of first cleavage and gastrulation at its new position (Freeman 1977, 1981a), indicating that in these animals, the embryonic and organismal (oral-aboral) axes are normally set up by the location of the female pronucleus, because that determines the site of first cleavage. The light-green arrow indicates the swimming direction of the animal.

meiotic reduction divisions generate polar bodies is defined as the animal pole of the primary (animal-vegetal) egg axis and, thus, can be used as a reference point for comparing the axial relationships of metazoan embryos. Although detailed information is lacking in many animal groups, in cnidarians (Eckelbarger et al., 2008), dipteran flies (Gilbert and Raunio, 1997), and echinoids (Frick and Ruppert, 1996; Frick et al., 1996) the animal pole normally corresponds to the position where the oocyte makes contact with its germinative epithelium, suggesting that the conditions for establishing axial embryonic polarity in these embryos are set up maternally. In virtually all investigated bilaterian embryos, fate mapping experiments have shown that subsequent development is organized along this primary egg axis (Goldstein and Freeman, 1997; Wall, 1990). For example, the site of gastrulation (the place where endoderm and/or endomesoderm is generated), the location of the mouth, head region, appendages, etc., are generated from predictable places corresponding to their position along the animal-vegetal axis. There are examples throughout the metazoan tree, including ctenophores, acoelomorphs, chordates, spiralians, and ecdysozoans, in which the stereotypy of embryonic development along the animal-vegetal axis allows the prediction of the exact fate of identified blastomeres (Gilbert and Raunio, 1997). Thus, consideration of the primary egg axis is likely to provide important landmarks for changes related to the evolution of developmental patterning.

When considering the role of the primary egg axis in the elaboration of body plans during early animal evolution, two taxa, ctenophores and cnidarians, are particularly relevant (Figure 1). Although poriferans (sponges) and placozoans (*Trichoplax*) branch near the base of the Metazoa (Figure 1), their body plans are difficult to compare with other metazoans, and their embryos, when present, are technically difficult to study. For example, adult sponges and placazoans do not display an obvious anterior-posterior axis either morphologically or behaviorally, and the developmental origin of germ layers (i.e., gastrulation) relative to the embryonic (i.e., animal-vegetal) axis or the adult body plan is not clear. Without these important details, these taxa are not likely to provide much additional insight into the evolution of bilaterian body plans. Ctenophores (e.g., comb jellies) and cnidarians, in particular, anthozoan cnidarians (e.g., sea anemones, corals, sea fans, and sea whips) with their simple life history, are important because they have a major longitudinal body axis and definitive guts that can be homologized to bilaterians. Furthermore, their early development can be studied in detail with relationship to their adult axial properties (Fritzenwanker et al., 2007; Lee et al., 2007; Martindale and Henry, 1999). All recent molecular phylogenomic studies agree that cnidarians are the sister group to all other bilaterians (Figure 1), and some suggest that ctenophores (not sponges) form the earliest branch in the metazoan lineage (Dunn et al., 2008). Even if the phylogenetic relationship of ctenophores relative to other metazoans is revised, the similarities in egg organization and axial properties of ctenophores and cnidarians as groups branching prior to the radiation of bilaterians suggest that something can be learned about the developmental basis

for the origin of ancestral character states from these groups of animals. These two uniquely distinct taxa could, therefore, bracket (Figure 1) important early events in metazoan evolution and provide insight into the developmental basis for body plan evolution.

Fate Mapping and the Relation of the Animal-Vegetal Axis to the Adult Axes in Ctenophores and Cnidarians

Vital dye labeling of defined regions/blastomeres in developing embryos allows one to predict the eventual fates of these regions in the resultant larval or juvenile adult body plan. Fate mapping experiments in cnidarians and ctenophore embryos have allowed three important facts to be defined. First, the animal pole (defined by the site of polar body formation) in both ctenophore (Freeman, 1977; Martindale and Henry, 1999) and cnidarian embryos (Freeman, 1981b; Momose and Schmid, 2006; Schlawny and Pfannenstiel, 1991; Tessier, 1931) is normally the site of the formation of the unipolar first cleavage furrow, which corresponds to the oral pole that gives rise to the future mouth of both adult ctenophores and cnidarian polyps (Figure 3). Furthermore, in both clades it has been shown experimentally that the site of first cleavage is causally involved with the formation of the oral-aboral axis (Freeman, 1977, 1981b). If the zygotic nucleus is moved from the original animal pole to an ectopic site by gentle centrifugation, a new oral-aboral axis is established, with the new site of first cleavage determining the future oral pole (Figure 3). Drug treatments that generate two simultaneous cleavage furrows in cnidarian embryos generate two mouths, indicating that the site of first cleavage plays an important role in organizing the future oral opening (Freeman, 1981a). These experiments show that although there might be a consistent relationship of the primary egg axis to future developmental events that are set up maternally, they merely establish the conditions for the formation of the first cleavage furrow. Thus, unlike most other bilaterians, both the definitive embryonic and organismal axial properties of ctenophores and cnidarians are not irreversibly established maternally (Goldstein and Freeman, 1997), but are set up as an active consequence of the developmental program (e.g., the site of first cleavage).

Fate mapping experiments show that the single opening to the cnidarian and ctenophore gut arises from the same region of the embryo (animal hemisphere) that forms the mouth in all other bilaterians (Goldstein and Freeman, 1997; Henry et al., 2001; Holland and Holland, 2007; Nielsen, 1999, 2005a). This suggests that the oral pole of adult ctenophores and cnidarians is homologous to the anterior pole of bilaterians and that the single opening is homologous to the mouth of other bilaterians. This relationship is also supported by molecular data. Recent work has shown that the same genes used to argue for the homology of the mouth in protostomes and deuterostomes (Arendt et al., 2001) are also expressed in the single mouth opening of an acoel flatworm (Hejnol and Martindale, 2008a) and in the oral openings of cnidarians (Martindale et al., 2004; Scholz and Technau, 2003) and ctenophores (*brachyury* only, Yamada et al., 2007). Thus, the mouths of all animals appear to be homologous, with the possible exception of the chordates (Figure 1), which might have evolved a new mouth secondarily. The mouth of chordates does not express the same suite of genes other metazoans do (Christiaen et al., 2007; Yasui and Kaji, 2008), and its position forms independently of a circumoral component of the nervous system shared by most other metazoans (Lacalli, 2008). It has been suggested that the chordate mouth arises by lateral modifications of the pharyngeal apparatus of early cephalochordate-like ancestors (Lacalli, 2008; Yasui and Kaji, 2008). That the mouth is homologous in all (nonchordate) metazoans seems functionally plausible. If the oral openings in different evolutionary lineages arose independently, one would have to argue for an ancestor with a gut cavity, but no mouth, which seems unlikely. If this interpretation is correct, the single mouth/anus of ctenophores, cnidarians, and acoels preceded the evolution of the through gut, suggesting that the anus arose independently of the mouth in protostome and deuterostome lineages. This observation argues against hypotheses that assume the mouth in ancestral protostomes and deuterostomes evolved independently and that the bilaterian mouth and anus evolved simultaneously from a common opening (Figure 2).

The Ancestral Metazoan Mouth Was Never Located at the Posterior Pole and the Neural Structures of Cnidarians and Ctenophores Are Not Homologous to the Bilaterian Brain

The primary argument supporting the idea that the mouth of ancestral metazoans formed at the posterior pole and moved anteriorly (Figure 2) is the observation that the future mouth of cnidarians forms at the trailing edge of the ciliated planula stage. It is widely assumed that the leading edge of the swimming planula stage, with its sensory apical ciliary tuft, corresponds to the anterior pole of the bilaterian anterior-posterior axis and that the apical sensory organ is homologous with the bilaterian brain (Nielsen, 2008). Although both ctenophores and cnidarians have specialized neural structures derived from cells born at their aboral pole, we argue that these organs are neither homologous to bilaterian neural structures nor are they homologous to one another. Ctenophores have a gravity sensing statocyst called an apical organ consisting of $CaSO_4$ containing lithocytes perched on balancing cilia, while cnidarian planula have chemosensory cells in an apical tuft. Although these two structures (apical organ and apical tuft) are derived from the same embryonic region, they are radically different in structure and function and are not considered homologous to one another (Ax, 1996; Scholtz, 2004). Further evidence that the neural structures in ctenophores and cnidarians are unlikely to be homologous with any anterior bilaterian neural structure is their formation in a completely different region of the embryo. Bilaterian anterior neural structures form from derivatives of the animal pole, while the apical organ and apical tuft of ctenophores and cnidarians develop from derivatives of the vegetal pole (Figure 3). Furthermore, ctenophores and cnidarians do not express some conserved molecular markers for anterior bilaterian brain development in their apical neural center (such as *BF-1*, *otx*, or paired-class genes) (de Jong et al., 2006; Matus et al., 2007a; Pang and Martindale, 2008; Yamada and Martindale, 2002). Although more information, particularly with respect to the molecular basis of neuronal determination in ctenophores, is needed, there is as of yet little morphological or molecular evidence to argue for the homology of either the ctenophore apical organ or the cnidarian apical tuft to the bilaterian anterior brain.

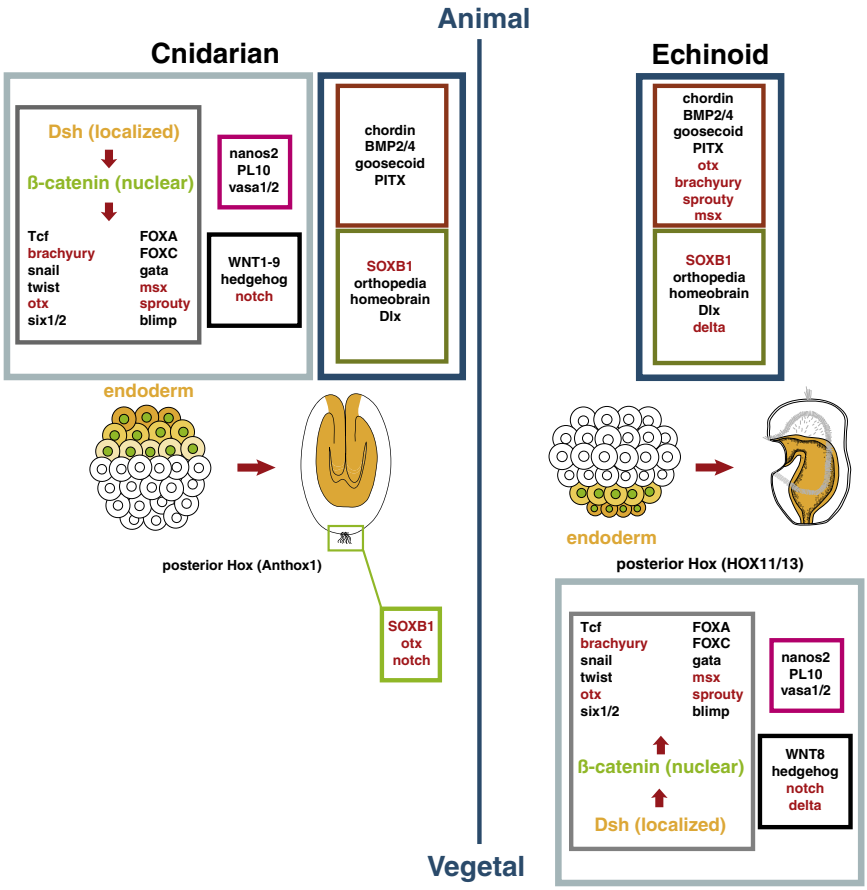

Figure 4. Translocation of Regulatory Gene Expression during the Evolutionary Change of the site of Gastrulation

Subset of genes expressed in derivatives of either the animal or vegetal hemisphere in the cnidarian *Nematostella* (left column) and echinoid deuterostomes (*Strongylocentrotus*, *Paracentrotus*) (right column). The animal pole is situated toward the top of the page; the vegetal, toward the bottom. Genes associated with oral ectoderm (brown box) and neural determination (green box) are expressed in derivatives of the animal hemisphere in both groups. Genes associated with gut development (gray box), germ line development (purple box), and signaling molecules associated with the blastopore (black box) have largely changed their position of expression due to changes in the local stability of Dsh (gold shading) and β-catenin (green nuclear localization) at the animal (*Nematostella*) and vegetal (echinoid) pole. Some genes (red print) are expressed at both poles, presumably due to multiple *cis* regulatory inputs.

These data are significant as they refute evolutionary scenarios that focus on the swimming direction as the indicator of the homology of the anterior-posterior body axis. If "anterior" in the planula stage of the cnidarian life cycle is defined by the direction of swimming, then the argument that the mouth of ancestral metazoans formed at the posterior pole may be flawed. Because the apical tuft is not likely to be homologous to anterior sense organs in other bilaterians (Hyman, 1951; Salvini-Plawen, 1978), and the mouth of cnidarians and ctenophores is generated from the same embryonic region and expresses the same set of molecular markers as other bilaterians, it is more parsimonious to argue that cnidarian larvae swim "backward," with their presumptive mouth at the trailing end (Figure 2). It should be noted that ctenophores, which lack a larval stage, swim with their mouth forward (although they are also capable of ciliary reversal), so aboral sense organs can form at either the leading (cnidarian) or trailing (ctenophore) ends in these two metazoan groups. Thus, swimming direction in these two taxa is of little use for determining the homology of ancestral symmetry and axial properties. The apical tuft of cnidarian larvae seems to be a specialization co-opting neural cell types for the dispersal phase of the life history of this group. Recent molecular data has suggested that the larval apical organs of protostomes and deuterostomes (both located in the vicinity of the adult brain) appear to have evolved independently (Dunn et al., 2007). These data suggest that although the adult brain of most bilaterians might be homologous and form in the anterior region, new neural structures can evolve independently during larval periods in different metazoan groups. Thus, the fate maps predicted by the animal-vegetal axis appear to be better predictors of organismal polarity and body plan organization than larval swimming direction.

The Change of the Site of Gastrulation and Its Relation to the Bilaterian Mouth

Arguably, the most important result of fate mapping experiments in ctenophores and cnidarians is that the site of gastrulation and the origin of germ layer (endoderm/endomesoderm) formation both occur at the animal pole. Ctenophores generate definitive muscle cells from a lineage of micromeres born at the animal pole (Martindale and Henry, 1999), and their sister cells generate the endodermal portion of the gut (Figure 3). Thus, in ctenophores germ layer formation occurs where the mouth is formed at the animal pole. Cnidarians do not make definitive muscle cells, but molecular studies of germ layer formation in anthozoans show that virtually all of the genes involved in endomesoderm formation in bilaterian embryos, including the core genes (*otx*, *Gata*, *Foxa*, *brachyury*, *blimp*, and *notch/delta*) identified as components of an evolutionary conserved endomesodermal ("kernel") gene regulatory network (Hinman et al., 2007) (Figure 4), are expressed in cnidarian epithelial tissue that lines the gastric cavity or the pharynx that leads to the oral opening (Martindale et al., 2004; Matus et al., 2006; Mazza et al., 2007; Scholz and Technau, 2003). This conservation in components of the endomesodermal gene regulatory network provides compelling evidence that the endodermal and pharyngeal tissue of cnidarians (and presumably ctenophores) is homologous with that of the gut and oral ectoderm of bilaterians and that both endoderm and mesoderm of bilaterians evolved from an ancestral endomesodermal layer. Fate mapping experiments have shown that the definitive endoderm is generated from the oral pole/animal hemisphere (Fritzenwanker et al., 2007; Lee et al., 2007) in both anthozoan cnidarians and ctenophore embryos.

Thus, one of the fundamental differences between cnidarians and ctenophores on the one hand, and bilaterian embryos on the other, is the position of the site of gastrulation (i.e., endoderm/endomesoderm formation) relative to the primary egg axis. In most bilaterian embryos, endoderm formation and the site of gastrulation occurs at the vegetal pole, not the animal pole (Gilbert and Raunio, 1997; Siewing, 1969). The transition of the site of gastrulation from the animal to the vegetal pole must have occurred in the bilaterian stem lineage, as fate mapping experiments in acoel flatworms show that endomesoderm arises from vegetal macromeres (Henry et al., 2000). In acoels, the mouth does not form from the site of gastrulation (Hejnol and Martindale, 2008a), and therefore hypotheses that suggest a direct connection between the site of gastrulation and the mouth in acoels (e.g., Hyman, 1951) are not correct. The realization that the mouth forms independently of the blastopore and that this separation happened in the stem lineage of the Bilateria helps explain the large variation in the positional relationship of the oral opening to the site of gastrulation in bilaterian embryos (Hejnol and Martindale, 2009; Lankester, 1877; Salvini-Plawen, 1980).

Cnidarians and Ctenophores: The Only "True" Protostomes?

In deuterostomes, the site of gastrulation occurs at the vegetal pole and clearly gives rise to the anus. Protostomia is a clade of animals that includes two diverse groups called lophotrochozoans and ecdysozoans (Figure 1). These two groups are supposed to be united by the feature that the site of gastrulation (blastopore) becomes the mouth (protostomy), but their gastrulation is much more variable (Hejnol and Martindale, 2009). In fact, rarely has the mouth been described to originate from derivatives of the vegetal pole, and thus the blastopore, which forms at the vegetal pole, does not give rise to the mouth in most protostomes. Instead, embryonic fate mapping and the phylogenetic topology suggest that mouth formation from oral ectoderm from the animal hemisphere is ancestral in both protostomes and deuterostomes and that the endoderm forms at the vegetal pole. In protostomes, the anus, when present, forms independently of the site of gastrulation. For example, in the large group of protostome animals (Figure 1) that display spiral cleavage (e.g., annelids, mollusks, and nemerteans), the anus forms in the ectodermal territory derived from the dorsal side of the embryo, not the site of gastrulation at the vegetal pole (Nielsen, 2004, 2005b). Thus, neither the mouth nor the anus directly corresponds to site of gastrulation (i.e., derivatives of the vegetal pole) in most protostomes. Therefore, cnidarians and ctenophores seem to be the only animal groups in which there is direct evidence that the position of the blastopore and mouth occur in the same location, and because these two groups predate the origin of bilaterians, it is likely that they reflect the ancestral metazoan condition (Byrum and Martindale, 2004).

The realization that the ancestral metazoan mouth formed at the animal pole and that the site of gastrulation became dissociated from the position of the mouth in the bilaterian stem lineage challenges scenarios for the evolution of the bilaterian body plan that are based on a distinct relationship between the blastopore and the openings to the digestive system. The "Trochaea" hypothesis (Nielsen and Nørrevang, 1985) as well as the acoelplanuloid theory argue that both the site of gastrulation and original opening (mouth) to the gastric cavity corresponds to the posterior pole of modern day embryos (Figure 2). The amphistomy and bilaterogastraea concepts (Figure 2) suggest that the site of gastrulation/oral opening in early metazoans corresponds to the ventral surface of modern day bilaterians and that both the oral and the anal openings are derived from opposite ends of the blastoporal opening. Current evidence does not support these concepts. Not only do members of the bilaterian clade of acoelomorphs have a single oral opening (not two as predicted for the Urbilaterian), but the oral opening does not correspond to the blastopore (Hejnol and Martindale, 2008a; Henry et al., 2000). Furthermore, amphistomic gastrulation has never been shown to occur in any real organism (Hejnol and Martindale, 2009). These theories also do not explain the origin of bilaterality, because acoelomorphs are bilaterians with a single opening to the gut, and therefore bilaterality precedes the evolution of acoelomorphs. It has been argued based on the expression of developmental regulatory genes that cnidarians show bilateral symmetry, that symmetry was lost in some cnidarian groups, and that bilaterality might have been present in the cnidarian-bilaterian ancestor (Finnerty et al., 2004; Matus et al., 2006).

Molecular Basis for the Change in the Site of Gastrulation

As we discussed above, while the position of the mouth has changed relatively little with respect to the primary embryonic (animal-vegetal) axis over evolutionary time, the most fundamental change in the bilaterian developmental program centers around the change in the site of gastrulation and the origin of endomesodermal tissues (Figure 2D). Molecularly, the site of gastrulation in echinoderms (Logan et al., 1999; Wikramanayake et al., 1998), a spiralian protostome (Henry et al., 2008), and the cnidarian *Nematostella* (Wikramanayake et al., 2003) are all determined by the site of nuclearization of the bifunctional protein β-catenin, a gene known to be a downstream target of the Wnt signaling pathyway. Activation of β-catenin occurs at the vegetal pole in bilaterians and at the site of first cleavage (at the animal pole) in cnidarians, which, in both cases, are the sites of endomesoderm formation (Figure 4). Functional inactivation or destabilization of β-catenin results in the absence of endomesoderm, and stabilization results in excess endomesoderm (Henry et al., 2008; Logan et al., 1999; Wikramanayake et al., 2003; Wikramanayake et al., 1998). Could the site of nuclearization of β-catenin have changed 180° from the animal pole to the vegetal pole during bilaterian evolution? In echinoids, the site of gastrulation is determined by the maternal concentration of the protein Disheveled (DSH), which is associated with membranous vesicles anchored at the vegetal pole (Figure 4) (Ettensohn, 2006; Weitzel et al., 2004). In oocytes of the cnidarian *Nematostella vectensis*, DSH is preferentially localized to the membrane of the female pronucleus and is then transferred to the plasma membrane at the site of polar body formation and to the cleavage furrow at the site of first cleavage at the animal pole (Lee et al., 2007). The fact that DSH is associated with the membrane of the female pronucleus in cnidarians explains the results of Freeman (Freeman, 1981b) in which a new oral pole could be entrained by moving the position of the female pronucleus prior to first cleavage (Figure 3). Dominant-negative interference of DSH function in both cnidarians and sea urchins (echinoids) prevents the stabilization of β-catenin and gastrulation (Ettensohn, 2006; Lee et al.,

2007). Thus, the transition of the site of endomesoderm formation in metazoan evolution from the animal pole in cnidarians and ctenophores to the vegetal pole in all other bilaterians can be explained by the different asymmetric maternal localization of DSH protein. DSH is a good candidate for a protein that can change its spatial localization during development and over evolution because it contains known microtubule, microfilament, and phospholipid binding domains (Leonard and Ettensohn, 2007; Torres and Nelson, 2000; Weitzel et al., 2004). Evidence from other groups of cnidarians is consistent with a role for the WNT signaling pathway in establishing the oral-aboral axis (Momose et al., 2008; Momose and Houliston, 2007). Unfortunately, no information on the expression and localization of β-catenin or DSH is yet known in ctenophores, acoels, or other critical metazoan clades. It would be of interest to determine whether changes in protein structure are related to its cellular spatial stabilization in different lineages.

Possible Consequences of Changes in the Site of Gastrulation

There are a number of important consequences of changing the site of gastrulation from the animal pole in ctenophores and cnidarians to the vegetal pole in bilaterians. Some of these are practical and lead to testable hypotheses about the changes in architecture of conserved gene regulatory networks. Others are more conceptual but may provide a framework for understanding the variation in developmental patterning seen in different animal groups. Although it is dangerous to make broad generalizations from isolated cases, and it is far from clear which of the handful of examples available are the most appropriate to compare, as an exercise we compared gene expression domains between an anthozoan cnidarian (*Nematostella vectensis*) and an echinoid deuterostome (a bilaterian) reconstructed based on studies in several different sea urchin species (Figure 4). It will be important to include information from other phylogenetically relevant organisms such as hemichordates, spiralians, and a variety of marine ecdysozoans to gain insight into the early diversification of developmental patterning mechanisms.

Gene expression studies in *N. vectensis* have identified a relatively large number of genes with expression patterns spatially or temporally restricted to derivatives of the animal pole, the vegetal pole, or in derivatives of both opposing poles during different stages of development (Figure 4). For example, genes associated with the bilaterian oral ectoderm (Matus et al., 2006) and anterior nervous system (Marlow et al., 2009) are expressed in derivatives of the animal hemisphere in both bilaterians and *N. vectensis* (Figure 4). Genes known to be associated with the endomesodermal circuit in derivatives of vegetal cells in bilaterians (Figure 4) are expressed in cells derived from the animal pole in *N. vectensis* (Martindale et al., 2004; Scholz and Technau, 2003).

We argue that the positional shift in β-catenin stabilization and nuclearization from the animal pole in cnidarians (and maybe ctenophores) to the vegetal pole in bilaterians drove, either directly or indirectly, the expression of many endomesodermal cell fate-determining genes to derivatives of the vegetal pole. Several genes associated with germ line development (Figure 4) also changed their position of expression and these are known to be correlated with nuclear β-catenin localization in urchins (Voronina et al., 2008). However, many other genes associated with formation of bilaterian oral ectoderm (e.g., *goosecoid*, *brachyury*, *chordin*, *otp*, *BMP2/4*) remained associated with the animal pole (Arendt et al., 2001; Hejnol and Martindale, 2008a), supporting the homology of the mouth in metazoan evolution. While we predict that not all described genes will follow these simple changes in spatial localization, the functional link between these genes can be investigated using bioinformatic (e.g., common *cis* regulatory architecture) and misexpression/knockdown approaches. Intra-taxon comparisons in echinoderms have already shown variation in the recruitment of genes into regulatory networks that lead to changes in morphological patterning (Hinman and Davidson, 2007). Broader taxon sampling of molecular studies is clearly required to understand the plasticity of such networks and their role in patterning evolutionary novelties.

Genes Expressed at Both Poles of the Embryo

Virtually all developmental regulatory genes are controlled by a complex interaction of multiple *trans*-acting factors integrated by their *cis* regulatory architecture (Davidson, 2006). Genes, such as *otx*, which are expressed in multiple, highly conserved developmental domains (e.g., neural and endomesodermal derivatives) have multiple *trans*-activating factors that control expression in distinct domains. For example, when β-catenin became activated in a distinct set of derivatives of the vegetal hemisphere early in bilaterian evolution, it drove *otx*, *foxA*, and *brachyury* expression (Figure 4), while other (currently unidentified) *trans*-acting factors remained expressed in the animal hemisphere to drive ectodermal fates. The expression of a few genes (e.g., *brachyury*, *otp*, *hedgehog*, *foxA*) associated with both anterior and posterior domains of the gut in some animals has been used as one of the strongest arguments for the process of amphistomy (Arendt, 2004; Arendt et al., 2001). These results can now be reinterpreted in light of the change in the site of gastrulation. These genes persist in their ancient position due to their role in oral (animal pole) ectodermal patterning but are also expressed with respect to their new role in endomesodermal patterning, downstream of the stabilization of nuclear β-catenin at the vegetal pole. Since the mouth is unlikely to have formed from the blastoporal opening in the bilaterian ancestor, amphistomy is probably not the basis for body plan evolution (Hejnol and Martindale, 2008a, 2009).

Organizing Centers in Development

The blastopore is not only the site in the embryo where endomesodermal fates are being born, but it often possesses "organizing ability" that patterns axial properties of the embryo. For example, when transplanted to ectopic locations, blastoporal cells can induce duplicated anterior-posterior axes. The dorsal lip of the blastopore is well known to have morphogenetic ability in vertebrate embryos (Spemann and Mangold, 1924). Vegetal organizing centers associated with the site of gastrulation are also well known for echinoids (Hörstadius, 1935) and spiralians (Clement, 1986). Recent work has shown that the same properties exist in cells making up the blastopore in cnidarian embryos. If cells around the lip of the *N. vectensis* blastopore are transplanted to a distant site, a second oral-aboral axis is established that recruits neighboring nontransplanted cells (Kraus et al., 2007). Thus, blastoporal organizer activity in cnidarians is comparable to that seen in bilaterian embryos.

Work with bilaterian embryos indicates that the cell-cell signaling activity of organizing centers is mediated by the

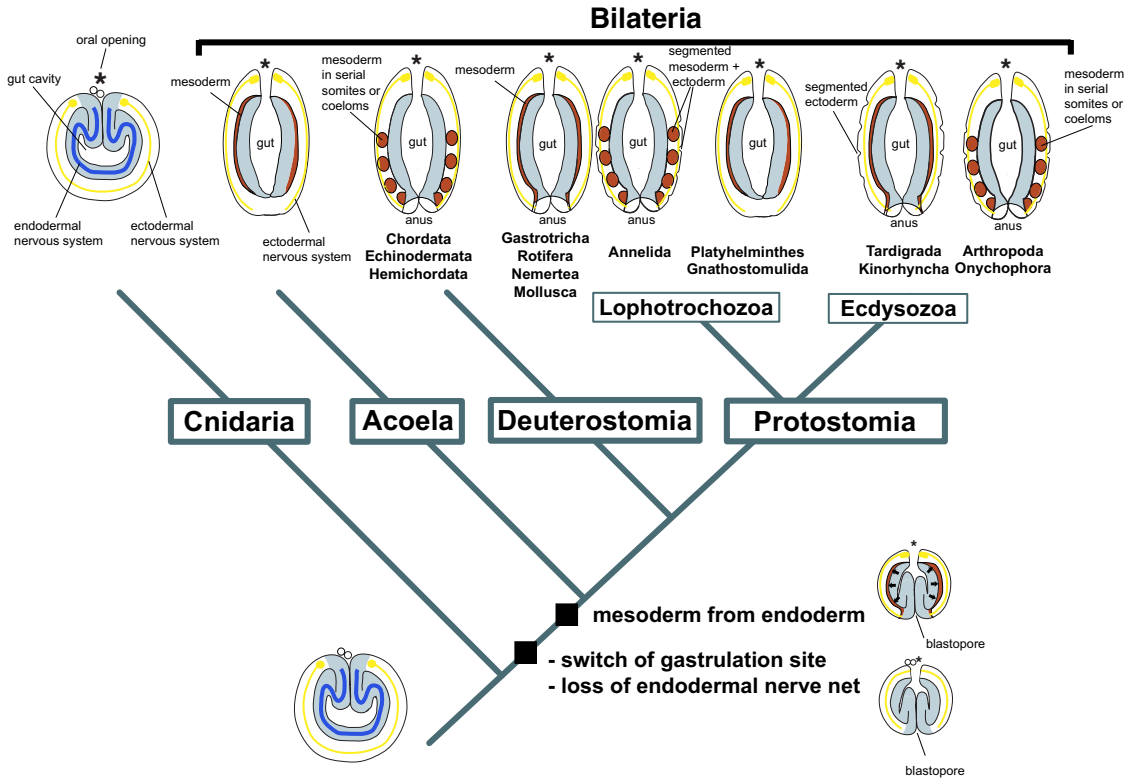

Figure 5. Testable Scenario for the Evolution of the Main Features of Body Organization

The switch of the site of gastrulation to the vegetal pole led to the loss of the endodermal nervous system (dark-blue structure in Cnidaria), while derivatives of the animal pole elaborated the ectodermal nervous system (yellow). In the Bilaterian lineage, the mesodermal germ layer (brown) evolved from the bifunctional endoderm (see cnidaria). The connection of the process of tissue formation to an oscillating gene network of the vegetal (posterior) pole of the bilaterian animals (e.g., Notch/Delta) led to the convergent formation of repeated structures in some, but not all, animal groups.

regulation of a relatively small number of diffusible ligands, such as BMP2/4 and BMP5-8 (Matus et al., 2006; Rentzsch et al., 2006), hedgehog (Matus et al., 2008), and FGF8/17/18 (Matus et al., 2007b; Rentzsch et al., 2008); a large number of wnt family members (Kusserow et al., 2005) are also associated with the blastopore in *N. vectensis* (Figure 4) and are thus excellent candidates for mediating the organizing activity of the cnidarian blastopore. Therefore, when the site of gastrulation changed from the animal pole to the vegetal pole in early bilaterian evolution, the expression of these diffusible ligands was also inherited by vegetal endomesodermal descendents. Some of the genes, such as Wnts (Bischoff and Schnabel, 2006; Yu et al., 2007) and FGF genes, likely became involved in anterior-posterior patterning secondarily in distinct evolutionary lineages (Holland, 2002). The staggered expression of Wnt genes along the oral-aboral axis in both ectodermal and endodermal tissues in *N. vectensis* following gastrulation suggests roles for these genes in axial patterning (Kusserow et al., 2005). Changing the site of gastrulation therefore has more profound effects on organismal patterning than merely changing the position of endoderm/endomesoderm formation (Figure 5).

Protracting the Developmental Period: Terminal Addition

The site of gastrulation is where important developmental decisions are made that give rise to new mesodermal and endodermal tissues. In virtually all bilaterians, there is an anterior to posterior gradient in development, meaning that the tissues in the anterior region are born earlier and continue to develop as new tissues are being generated in the posterior region. If the site of gastrulation moved from the anterior pole to the posterior region (derived from more vegetal regions), and the developmental period was extended, the body could continue to grow in size posteriorly while the anterior region began its differentiation program, e.g., in form of a growth zone (Figure 5). In vertebrates, new tissues are generated posteriorly in the presomitic segmental plate. In many marine invertebrates, such as the trochophore-like and naupliar larvae characteristic of lophotrochozoans and arthropods (crustaceans), respectively, the oral/pharyngeal region and anterior neural structures become functional while posterior body regions are still being generated from a subterminal growth zone (Jacobs et al., 2005). In some extreme cases, such as annelids, new body segments are continually generated throughout the life of the organism. While formation of new tissues might not occur through the same morphogenetic process (e.g., via a blastopore) during these later developmental stages, they may arise by molecular mechanisms similar to those deployed during the embryonic period. This spatial and temporal heterogeneity in cell fate specification might promote functional specialization, giving rise to morphologically distinct regions of the body along the anterior-posterior axis. For example, the anterior-most regions of the developing

organism could specialize early into differentiating sensory and feeding structures, middle regions into swimming or walking appendages, while more posterior regions that are generated later would be dedicated to reproduction or gamete production.

It should be noted that this posterior positioning of new tissue production could be exploited independently in different animal lineages by coupling it to a oscillatory gene network, such as the *notch/delta/hes* (Pourquie, 2003; Tautz, 2004), because the cell signaling pathways and tissue-specific networks of gene regulation were already present in their new location prior to the appearance of any complex bilaterian morphological traits (Figure 5). It is therefore not necessary to invoke a complex segmented urbilaterian ancestor, because serially repeated structures such as somites, nephridia, ganglia, ectodermal annuli, etc., could have arisen independently in different lineages using the toolkit genes that were already present in an unsegmented ancestor (Figure 5). Indeed, a close examination of the terminal patterning mechanisms in different animal lineages reveal significant differences in the cellular and molecular details of patterning, despite the fact that they utilize largely overlapping molecular pathways (Pourquie, 2003; Seaver and Kaneshige, 2006; Siewing, 1969). For example, segmental patterning is driven by mesodermal tissues in vertebrates but by ectodermal tissues in arthropods (Schmidt-Rhaesa, 2007).

The Evolution of the Nervous System

One of the unique features of cnidarians is that they have both ectodermal and endodermal nerve nets (Hertwig and Hertwig, 1879). The existence of endodermally derived neurons is unknown in bilaterian animals (Figure 5). In a recent study of the organization of the nervous system of *N. vectensis*, it was found that the ectoderm possesses a variety of sensory neurons (including the stinging cells called cnidocytes), but all of the ganglion cells (the neurons that make connections with other neurons in the nerve net) are located in the endoderm (Marlow et al., 2009). As has been pointed out repeatedly in this review, endoderm in cnidarians is generated from cells derived from the animal (oral) pole, and the largest concentration of diverse neural cell types in cnidarian polyps is associated with the pharynx in oral and pharyngeal nerve rings (Fautin and Mariscal, 1991). Anterior neural structures (''brains'') in virtually all bilaterians studied are derived from cells originating at the animal pole (Gilbert and Raunio, 1997). One consequence of the change in the site of gastrulation in bilaterian animals is that expression of many of the nuclear β-catenin-mediated components of the endomesodermal determination network became spatially distinct (vegetal pole) from ectodermal animal hemisphere tissue, which continued to express oral and neural patterning genes (Figure 4). We argue that this spatial separation of these two tissue types could allow the oral ectoderm and networks of gene activity associated with neural differentiation (Figures 4 and 5) to remain active in derivatives of the animal hemisphere and endomesodermal gene expression to operate in derivatives of the vegetal pole. Presumably, the elaboration and consolidation of neural tissues in anterior regions would evolve more rapidly if cells were not also competing and participating with endomesdodermal cell fates in the same tissue. The initial stages of this antagonism might be mediated by the interaction of *SoxB1* and the canonical β-catenin pathway. Inhibition of the β-catenin pathway in echinoids leads to the expansion of *SoxB1* expression and the promotion of neural cell fates (Kenny et al., 2003). Translocation of β-catenin stabilization in the animal pole of cnidarians to the vegetal pole of bilaterians would reduce *SoxB1* antagonism in animal hemisphere descendents and promote neurogenesis in anterior cells. Consistent with this interpretation, there is no evidence for an enteric nervous system associated with the vegetally derived gut in acoelomorph flatworms (Rieger et al., 1991). Thus, early in bilaterian evolution, neural fates remained associated with animal hemisphere derivatives when endomesodermal fates relocated to the vegetal pole, and reintegration of the nervous system by innervation of endodermal tissues (i.e., the enteric nervous system) occurred later in bilaterian lineages (Figure 5).

It should be noted that many of the genes that show multiple domains of expression are those expressed in the aboral apical tuft (Marlow et al., 2009; Rentzsch et al., 2008). These genes (e.g., *SoxB1*, *netrin*, *Notch*, *FoxQ*, and *FGF8/17/18*), which presumably promote neuronal differentiation, are also expressed in the heavily neuralized pharynx (Marlow et al., 2009). Thus, additional studies might reveal how this putative neural gene regulatory cassette was co-opted over evolutionary time to generate sensory structures in derivatives of the vegetal pole in cnidarian and ctenophore embryos.

Summary/Conclusions

Most theories of body plan evolution have depended on examining changes in morphology from the organismal perspective (e.g., direction of locomotion, position of the mouth relative to the substrate). Here, we present a view from the embryological perspective, using fate mapping and new molecular information to show that changes in developmental processes relating to gastrulation events provide mechanistic insight into body plan organization. In particular, the spatial separation of endomesodermal gene regulatory networks from oral and neural embryonic domains by changing patterns of stabilization of β-catenin has allowed each domain to diversify in form and function. The endomesodermal gene regulatory network includes not only genes involved in endodermal and mesodermal specification, but also those genes responsible for cell signaling events that help to ''organize'' subsequent developmental events and drive life history evolution. These data present testable hypotheses for learning more about the interaction and evolution of gene networks during embryonic development.

Further details of the gene regulator networks that trigger the separation of the mesoderm from the endomesoderm and specify the position of the deuterostome mouth are particularly important to understanding body plan reorganization and the evolution of novel cell types. With a better understanding of the relationships among extant animals, it should be possible to map key transitions in developmental mechanisms that have led to the diversification of biological form.

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